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A new species of *Gordius* (Nematomorpha, Gordiidae) from the karstic caves in the Wuling Mountains, Central China

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Abstract

Gordius wulingensis **sp. nov.**, a newly described horsehair worm, parasitizes *Tachycines* (Orthoptera, Rhaphidophoridae). Free-living specimens are mainly found in karstic cave puddles. In recent years, a total of 37 creamy white horsehair worms were collected from various caves in the Wuling Mountains. Characteristic for the species is that males exhibit concentrated bristles at the tips of the inner lobes and scattered bristles along the posterior half of the caudal lobe. The inner wall of the cloacal opening displays honeycombed areoles. The body cuticle contains dense bristles in the anterior and posterior ends; the density of bristles is decreasing in the mid-body. Distinct longitudinal sharp ridges are visible on the ventral and dorsal surfaces. Adults are present in caves year-round, while the entire life cycle is completed within the caves. In the laboratory, specimens can be kept alive for more than 3 months.

Key Words

Host, white horsehair worm, Wuling Mountains

Introduction

The phylum Nematomorpha is commonly known as horsehair worms, hairworms, gordian worms, or simply gordiids (Carvalho 1942; Hanelt et al. 2005; Schmidt-Rhaesa 2013; Anaya et al. 2019). Most species are free-living as adults in freshwater environments, but one has a terrestrial life cycle (*G. terrestris*, Anaya et al. 2019). Juveniles are parasites in multiple terrestrial arthropods (Townsend 1970; Hanelt et al. 2005; Anaya et al. 2019). During the parasitic phase, horsehair worms absorb nutrients via their integument, free-living adults do not feed, and nutrient absorption is confined to the juvenile stage (Schmidt-Rhaesa 2013).

Gordiids have been identified as one of the most understudied groups of parasites (Poulin 1998) because of their complex life cycle, challenging general ecology for study, and relatively limited diagnostic characters (Hanelt et al. 2005; Schmidt-Rhaesa 2010). Despite significant contributions from numerous scholars, the identification of many horsehair worm species remains challenging because their morphological features are difficult to discern through naked-eye observation and light microscopy alone, and systematic descriptions of their ultramicroscopic structures have been insufficient (Schmidt-Rhaesa 2010). Recent years have seen increased attention to the description and identification of horsehair worm eggs and larvae (Hanelt and Janovy 2002; Szmygiel et al. 2014; Chiu et al. 2020). Female horsehair worms can lay millions of eggs at a time (Anaya et al. 2021).

About 360-plus freshwater gordiid species are known globally (Schmidt-Rhaesa 2013; Yadav et al. 2017; Anaya et al. 2019). Up to now, 5 genera and 17 species have been recorded in China, including *Gordius amoyensis* Tang, 1934; *G. foochowensis* Tang, 1934; *G. omensis* Wu & Tang, 1933; *G. polychaetus* Tang, 1934; *G. chiashanus* Chiu, Huang, Wu, Lin, Chen & Shiao, 2020; *Acutogordius taiwanensis* Chiu, Huang, Wu & Shiao, 2017; *Chordodes formosanus* Chiu, Huang, Wu & Shiao, 2011; *C.* cf. *moutoni* Camerano, 1895; *C. tenoderae* Kirjanova, 1957; *C. wangi* Wu & Tang, 1933; *C. bipilus* Kirjanova, 1957; *C. caledoniensis* (Villot, 1874);

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Parachordodes sciacchitanoi Heinze, 1935; *Gordionus chinensis* (Villot, 1874); *G. kaschgaricus* Heinze, 1937; *G. wolterstorffii* (Camerano, 1888); and *Beatogordius chinensis* Schmidt-Rhaesa, 2011 (Schmidt-Rhaesa and Karraker 2017). Horsehair worms from mainland China and its surrounding islands are poorly documented, with many records being outdated and based on limited or single specimens (Schmidt-Rhaesa and Karraker 2017). The lack of a clear understanding of the resource base and diversity status of horsehair worms in China hinders their accurate identification and deeper investigation.

We describe here a new species of the genus *Gordius* from karstic caves in the Wuling Mountains, Central China. The definitive host was identified using worms with high sequence similarity collected from orthopterans of the genus *Tachycines*. Based on our field observations on the free-living adult worms and their hosts, along with the ecology of the cave, we suggest the possible life history of *Gordius wulingensis* sp. nov.

Materials and methods

Overview of the study region

Xiangxi Tujia and Miao Autonomous Prefecture of Hunan Province (approximately 109°10' to 110°22.5'E, 27°44.5' to 29°38'N, hereafter referred to as Xiangxi Prefecture) is situated on the eastern edge of the Yunnan-Guizhou Plateau, hinterland of the Wuling Mountains. It lies at the intersection of three major zoogeographical regions (Central China, South China, and Southwest China) within the upper Oriental boundary of China's zoogeographical division, covering an area of approximately 15,000 km^2. The region is characterized by a subtropical monsoon humid climate of suitable temperatures, abundant rainfall, dense river networks, widespread soluble carbonate rocks, well-developed karstic landforms, and numerous karstic caves (Wu 2002). These conditions provide favorable habitats for the development of cave biodiversity (Liu 2021; Tian 2023).

Collection and preservation of specimens

Five white horsehair worms were originally discovered by Professor Zhi-Xiao Liu from Jinji Cave in Yongshun County on 6 October 2014, but the specimens were lost. White horsehair worms were rediscovered in 2019, and our systematic investigation of them began in November 2022 (Table 1). A total of 13 female and 19 male free-living hairworms were collected from six locations in the karstic caves within the Xiangxi Prefecture. During the fieldwork, we focused on examining water pits and their surroundings inside caves, carefully turning over stones. All living worms were placed in water bottles filled with a small amount of water and transported back to the laboratory for further study. The worms were placed in a dark box designed to simulate the cave environment, with the temperature maintained at approximately 15 °C. Water from the cave was changed every five days. Insect hosts infected with horsehair worms were hand-collected from the caves. To find out whether a host was infected with worms, the posterior region was examined, and then the worms were collected by immersing the host in water or by host dissection (Fig. 7D, E). The infected host and the harbored worms were preserved in 95% ethanol for sequencing. Some of the samples were preserved in a solution of 75% ethanol for morphological examination. The specimens are deposited in the Hunan Wuling Mountain Biological Science Museum.

Morphological examination

Body length and maximum diameter of the worms were measured using a ruler and a vernier caliper. Morphological characteristics and behavioral habits were observed visually. Fragments (approximately 0.5 cm in length) of the anterior end, mid-body, and posterior end of the preserved samples were examined and photographed using a stereomicroscope (LEICA M205 C). Representative specimens were selected for scanning electron

Table 1. Basic information of karstic caves investigated in this study and the number and sex of horsehair worms found (in order of the time of discovery by caves). The first record from Jinji Cave is from Liu (2021); in this case, the sex was not identified.

Name of cave	City/County	GPS data	Elevation/m	No. and Sex of Gordiacea	Discovery time
Jinji Cave	Yongshun City	28°46'52"N, 110°14'6"E	910	5	2014/10/06
				5(4♂1♀)	2019/10/05
				4(2♂2♀)	2023/08/26
				4(3♂1♀)	2024/08/13
Jiuzhaiping Cave	Jishou City	28°17'17"N, 109°38'56"E	380	5(4♂1♀)	2022/11/19
				1(♀)	2023/08/24
				1(♂)	2024/04/10
				1(♂)	2024/04/25
Tangle Cave	Jishou City	28°19'11"N, 109°38'58"E	340	2(1♂1♀)	2022/11/25
				1(♀)	2023/08/24
				1(♀)	2024/08/30
Huangni Cave	Yongshun City	28°48'57"N, 110°14'19"E	1080	2(1♂1♀)	2023/05/02
				2(1♂1♀)	2024/08/14
Xiaodong Cave	Guzhang County	28°40'52"N, 109°56'52"E	320	2(1♂1♀)	2023/07/02
Baihu Cave	Jishou City	28°25'44"N, 109°44'31"E	250	1(♀)	2023/07/23
Total				37(19♂13♀5)	

microscopy (SEM) analysis. They were dehydrated using a series of ethanol and acetone solutions (95% and 100% ethanol (twice) and ethanol/acetone mixtures of 2:1, 1:1, 1:2, and 0:1), dried with a critical point dryer, coated by being sputtered with gold, and examined using a Zeiss Field Emission Scanning Electron Microscope (Sigma HD) at magnifications ranging from 100× to 15,000×.

COI gene sequencing and phylogenetic relationship analysis

Genomic DNA from adult horsehair worms was extracted using an Animal Genome DNA Extraction Kit (Tsingke, Changsha, Hunan). The partial cytochrome c oxidase subunit I (COI) sequence was amplified using universal primers (LCO1490 and HCO2198) (Folmer et al. 1994). PCR for both primer sets was initiated at 95 °C for 5 min, and amplification was conducted for 35 cycles of 95 °C for 1 min, 50 °C for 1 min, and 73 °C for 1 min, with a final extension at 73 °C for 5 min. We obtained high-quality COI sequences (>500 base pairs) from free-living individuals to be used in our phylogenetic analysis and estimates of intraspecific genetic distances.

The COI gene sequences obtained in this study for horsehair worms were submitted to the GenBank database for sequence alignment. Phylogenetic trees and genetic distance matrices were constructed using the maximum likelihood method implemented in MEGA11 (see detailed information in Table 2). We constructed a maximum likelihood phylogeny using IQ-TREE v2.2.0. First, sequences were aligned with MAFFT using the '--auto' strategy and normal alignment mode. Then, gap sites were removed with TRIMAl v1.2 using the "-automated1" command. Maximum likelihood phylogenies were inferred using IQ-TREE v under the TVM+R3+F model for 50,000 ultrafast bootstraps, as well as the Shimodaira–Hasegawa–like approximate likelihood-ratio test. Additionally, an uncorrected p-distance of COI was conducted in MEGA v.11.0.13.

Table 2. List of COI sequences obtained from GenBank for phylogenetic analyses in this study.

Accession number	Species/clade	Reference
KM382317	*Gordius* cf. *robustus* (Clade 8)	Hanelt et al. 2015
KM382316	*G.* cf. *robustus* (Clade 8)	Hanelt et al. 2015
KM382310	*G. terrestris*	Hanelt et al. 2015
KM382309	*G. terrestris*	Hanelt et al. 2015
KM382306	*G.* cf. *robustus* (Clade 6)	Hanelt et al. 2015
KM382305	*G.* cf. *robustus* (Clade 6)	Hanelt et al. 2015
KM382297	*G.* cf. *robustus* (Clade 5)	Hanelt et al. 2015
KM382296	*G.* cf. *robustus* (Clade 5)	Hanelt et al. 2015
KM382294	*G.* cf. *robustus* (Clade 4)	Hanelt et al. 2015
KM382293	*G.* cf. *robustus* (Clade 4)	Hanelt et al. 2015
KM382289	*G.* cf. *robustus* (Clade 3)	Hanelt et al. 2015
KM382288	*G.* cf. *robustus* (Clade 3)	Hanelt et al. 2015
KM382283	*G.* cf. *robustus* (Clade 2)	Hanelt et al. 2015
KM382282	*G.* cf. *robustus* (Clade 2)	Hanelt et al. 2015
KM382281	*G.* cf. *robustus* (Clade 1)	Hanelt et al. 2015
KM382280	*G.* cf. *robustus* (Clade 1)	Hanelt et al. 2015
KM382318	*G. attoni*	Hanelt et al. 2015
KM382319	*G. attoni*	Hanelt et al. 2015
KM382320	*G. balticus*	Hanelt et al. 2015
KM382321	*Gordius* sp. 2	Hanelt et al. 2015
KM382322	*G.* sp. 3	Hanelt et al. 2015
KM382323	*G.* sp. 5	Hanelt et al. 2015
KM382324	*G.* sp. 1	Hanelt et al. 2015
AB647235	*G.* sp. 7	Sato et al. 2012
AB647237	*G.* sp. 6	Sato et al. 2012
AB647241	*G.* sp. 8	Sato et al. 2012
KY172751	*G.* sp. 4	Tobias et al. 2017
KY172750	*G.* sp. 4	Tobias et al. 2017
KY172782	*G. paranensis* (Clade 2)	Tobias et al. 2017
KY172813	*G. paranensis* (Clade 2)	Tobias et al. 2017
KY172811	*G. paranensis* (Clade 1)	Tobias et al. 2017
KY172812	*G. paranensis* (Clade 1)	Tobias et al. 2017
KX591948	*Acutogordius taiwanensis*	Chiu et al. 2017
KX591947	*Acutogordius taiwanensis*	Chiu et al. 2017
MN784836	*G. chiashanus*	Chiu et al. 2020
MN784838	*G. chiashanus*	Chiu et al. 2020
PQ838977	JSUJIU05	This study
PQ838978	JSUJIN01	This study
PQ838979	JSUHUANG01	This study
Out group		
HM044105	*Chordodes formosanus*	Chiu et al. 2011
HM044124	*Chordodes formosanus*	Chiu et al. 2011
KY172780	*Euchordodes nigromaculatus*	Tobias et al. 2017
KY172803	*Euchordodes nigromaculatus*	Tobias et al. 2017
KY172747	*Parachordodes diblastus*	Tobias et al. 2017
KY172778	*Parachordodes diblastus*	Tobias et al. 2017

Results

Taxonomic account

Gordius wulingensis Zou & Liu, sp. nov.

https://zoobank.org/C004456B-EC0D-4E8B-BA87-0EB906CF4A7A

Type material. ***Holotype.*** • One male, China, Hunan Province, Wuling Mountains, Jiuzhaiping Cave (28°17'17"N, 109°38'56"E), ca. 380 m a.s.l., 19 November 2022, collected by Ya-Zhen Zou, Hunan Wuling Mountain Biological Science Museum (HWMBSM), voucher number: JSUJIU01.

Paratypes. • 1 ♂, same collection information as holotype, HWMBSM, voucher number: JSUJIU02. 1 ♀ and 2 ♂♂: same locality and collectors as holotype; collected on 24 August 2023 (HWMBSM, voucher number: JSUJIU03), 10 April 2024 (JSUJIU04), and 25 April 2024 (JSUJIU05).

Further specimens. • 1 ♂ and 1 ♀: China, Hunan Province, Wuling Mountains, Xiaodong Cave (28°40'52"N, 109°56'52"E), ca. 320 m a.s.l., 2 July 2023, collected by Zhi-Xiao Liu, HWMBSM, voucher number: JSUXIAO01, JSUXIAO02. • 1 ♀: China, Hunan Province, Wuling Mountains, Baihu Cave (28°25'44"N, 109°44'31"E), ca. 250 m a.s.l., 23 July 2023, collected by Zhi-Xiao Liu, HWMBSM, voucher number: JSUBAI01. • 1 ♀: China, Hunan Province, Wuling Mountains, Jinji Cave (28°46'52"N, 110°14'6"E), ca. 910 m a.s.l., collected by Shi Li and Yan Tang, 13 August 2024, HWMBSM, voucher number: JSUJINJI01.

•1 ♀: China, Hunan Province, Wuling Mountains, Huangni Cave (28°48'57"N, 110°14'19"E), ca. 1080 m a.s.l., collected by Shi Li and Yan Tang, 14 August 2024, HWMBSM, voucher number: JSUHUANG01.

Etymology. The specific epithet, "*wulingensis*", is derived from its habitat within the karstic caves in the Wuling Mountains.

Description of adult males (n = 5) (Figs 1–3, 5, 7). Body length 231.6 ± 77.51 (98–285) mm, width (widest, after dehydration) 0.45 ± 0.13 (0.22–0.53) mm, creamy white, smooth, with rainbow-like reflection (Fig. 7B, C).

The anterior end is columnar and spherical, with a nearly transparent white region between the epidermis and internal structures, and it lacks a dark-brown collar (Fig. 1A). Under SEM, the surface of the anterior end appears smooth (Fig. 1B) or wrinkled (Fig. 1C); short bristles (4.59 ± 1.89 (2–7.5) μm in length) are scattered except on the tip in most samples (Fig. 1D).

Several sharp ridges were seen on the dorsal and ventral sides (Figs. 2A; 7A). SEM reveals that the epidermis of the mid-body region in the male is smooth and lacks bristles, while the ventral region exhibits several ridges (Fig. 2B).

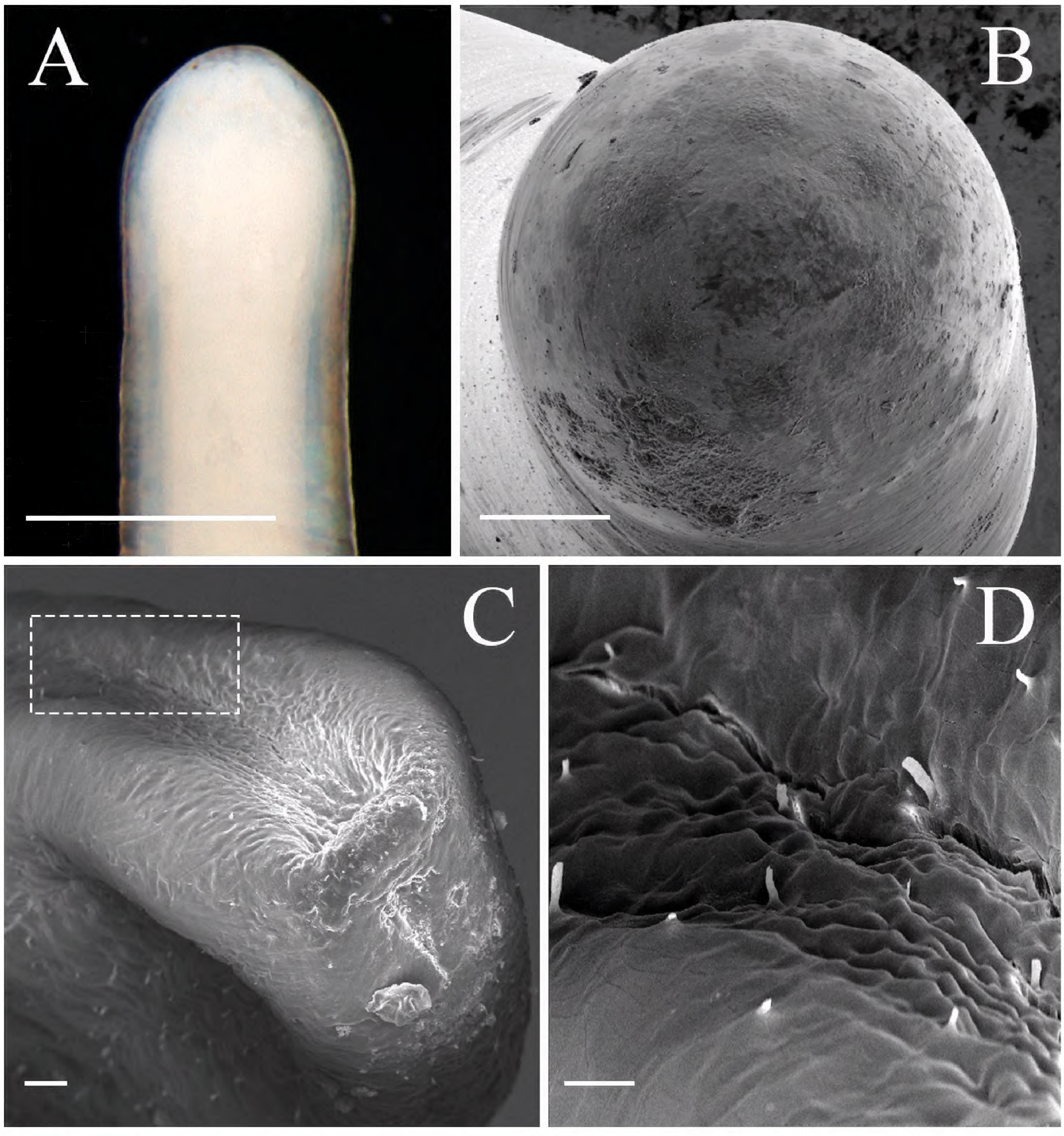

Figure 1. Anterior end of male *Gordius wulingensis* sp. nov. **A.** Stereomicroscopic image of the anterior end showing the white color and the lack of a dark-brown collar; **B, C.** SEM images of the anterior end surface that is (**B**) smooth with scattered short bristles and wrinkled; **D.** Close-up view of the dotted square in C showing the short bristles covered by a wrinkled structure. Scale bars: 1 mm (**A**); 100 μm (**B**); 20 μm (**C**); 10 μm (**D**).

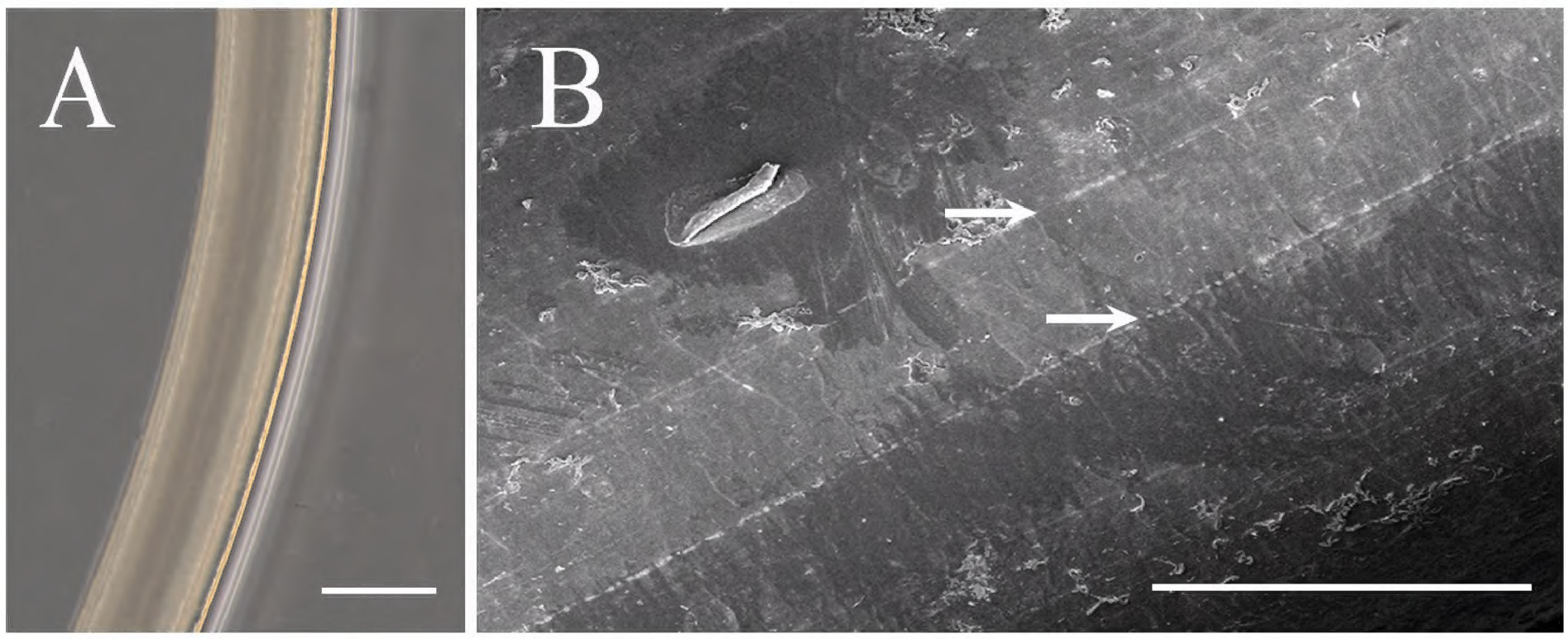

Figure 2. Mid-body of *Gordius wulingensis* sp. nov. **A.** Stereomicroscopic image of the anterior end showing several sharp ridges (**A**); **B.** SEM image of the anterior end showing sharp ridges. Scale bars: 100 μm (**A**); 500 μm (**B**).

The posterior end of males contains two tail lobes (Fig. 3A, B, C, E); each lobe is 384 ± 59.6 (300–449) μm long and 164.6 ± 33.8 (125–200) μm wide; length-to-width ratio is 2.41 ± 0.56 (1.50–2.84). Concentrated bristles are present at the tips of the inner lobes and scattered along the posterior half of the caudal lobe (Fig. 3B, C, D, E). The postcloacal crescent (Fig. 3A, B, C, E) is 243.4 ± 30.9 (200–280) μm long and 27 ± 10.4 (20–45) μm wide, short and roughly arc-shaped, located on ventral side near base of tail lobes. In the posterior end, the cuticle around the cloacal opening and the postcloacal crescent is pigmented browner than the surrounding cuticle, indicating a stronger cuticularization (Fig. 3A).

The cloacal opening is circular, 17.2 ± 5.1 (12–23.8) μm in diameter and anterior to postcloacal crescent (Figs. 3A, B, C, E). The wall inside the cloacal opening exhibits honeycombed areoles (Fig. 3F); no circumcloacal spines or bristles were observed in the region next to the cloacal opening.

Description of adult females (n = 5) (Figs 2, 4, 5, 7). Body length 429.2 ± 228(145–745) mm, width (widest, after dehydration) 0.65 ± 0.21(0.32–0.83) mm, creamy white, smooth. Under SEM, the anterior and posterior ends are columnar, rounded at their tips, and do not exhibit scattered bristles (Fig. 4A, C, E, H). The surface of the anterior end is smooth and may have concave infoldings, which probably are dehydration artefacts. Scattered short bristles, 4.70 ± 1.89 (2.81–7.35) μm in length, are present (Fig. 4B, D). The surface of the anterior end appears smooth (Fig. 4E) or wrinkled on the tip of one sample (Fig. 4H), with scattered short bristles, which are 6.11 ± 0.42 (5.81–6.4) μm in length (Fig. 4F, I). Notably, on the anterior end are scattered specialized spine-like bristles (indicated by the dashed box in Fig. 4E) (Fig. 4G). The head tapers, and the tail is rounded (Fig. 7A). The cloacal opening is small and circular (Fig. 4E, H).

Under SEM, the cuticle in the mid-body is smooth with rare bristles (Fig. 5C). In contrast, the density of bristles increases near the anterior and posterior ends of the body (Fig. 5A, B). Overall, bristles become progressively less common towards the mid-body.

Distribution, habitat. Based on our field collections and the historic specimens, *Gordius wulingensis* are mainly distributed in the karstic caves in the Wuling Mountain area of Hunan, China. This species can also be found in Yongshun County, Guzhang County, and Jishou City (Fig. 6). This species is usually observed in puddles, beneath stones, or sometime on the moist soil of the caves (Fig. 7C). Occasionally, they are found on the cave walls at elevations exceeding 1 m above ground level (Fig. 7D).

Phylogeny. This analysis used 22 terminal taxa of the genus *Gordius* and three out-group species to build molecular phylogenetic trees (Table 3, Fig. 8). The average length of the COI gene was 668 bp, with a maximum of 692 bp and a minimum of 554 bp. The genetic distances between COI sequences of *Gordius wulingensis* specimens collected from different caves ranged from 0.000 to 0.012. Due to these minimal genetic distances, we concluded that they represent the same species. The mean interspecific genetic distances between *Gordius wulingensis* and other *Gordius* species or clades were in the range of 0.162–0.264 (Table 3). No subgroup was detected because the polytomic topology exhibited low bootstrap values and short genetic distances. *G. wulingensis* clusters closest with *G. paranensis* and *G.* sp. 5 and 6.

Diagnosis. All specimens exhibit a rainbow-like reflection on the skin, white body color, lacking of a dark-brown collar. Distinct longitudinal sharp ridges visible on the ventral and dorsal surfaces. Males exhibit concentrated bristles at the tips of the inner lobes and scattered bristles along the posterior half of the caudal lobe, extending downward to the same length. Honeycombed areoles present on the inner wall of the cloacal opening. Dense bristles in the anterior and posterior ends, decreasing in the mid-body. Immature worms found in *Tachycines* sp. (collected on 25 April 2024), species identity confirmed by COI gene.

Figure 3. Posterior end of male *Gordius wulingensis* sp. nov. **A.** Stereomicroscopic image of the posterior end; note the brownly pigmented postcloacal crescent and cloacal opening; **B–E.** SEM images of (**B, C**) an overview of the posterior end with bristles scattered on the (**D**) lobe tips (C arrow), and (**E**) concentrated on the inner side of the lobe tips (arrows); **F.** Cloacal opening with areoles on the inside wall. Scale bars: 1 mm (**A**); 100 μm (**B, C, E**); 50 μm (**D**); 10 μm (**F**).

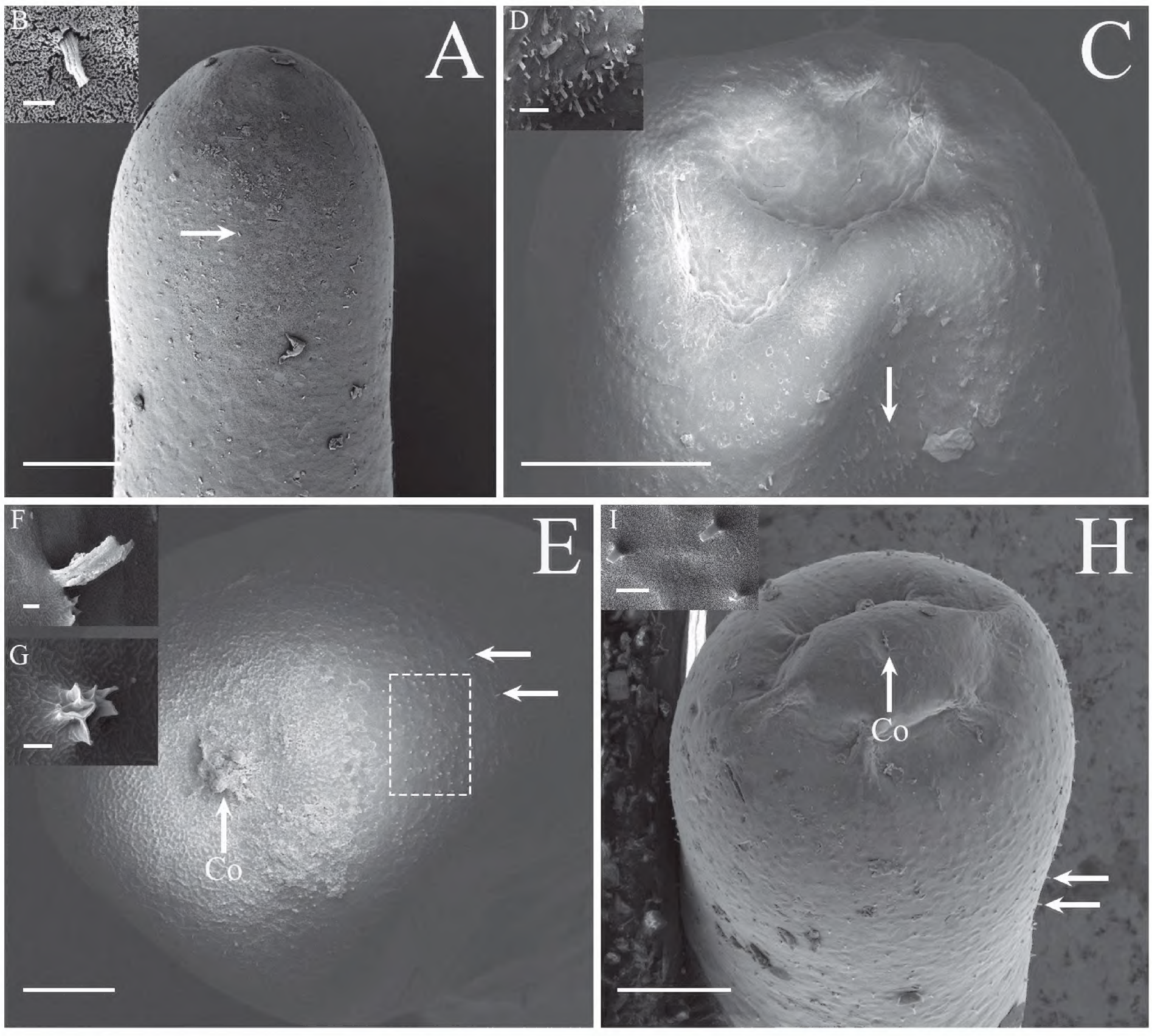

Figure 4. Female *Gordius wulingensis* sp. nov. **A, B.** SEM images of (**A**) cuticle in the smooth anterior end with scattered short bristles (arrows) and (**B**) close-up view of a short bristle; **C, D.** SEM images of (**C**) cuticle in the concave anterior end (may dehydration artefacts) with scattered short bristles (arrows) and (**D**) close-up view of a short bristle; **E, F, G.** SEM images of (**E**) cuticle in the smooth posterior end with scattered short bristles (arrows) and (**F, G**) close-up view of a short bristle; **H, I.** SEM images of (**H**) cuticle in the wrinkled posterior end with scattered short bristles (arrows) and (**I**) close-up view of a short bristle; Co: cloacal opening. Scale bars: 100 μm (**A, C, E, H**); 10 μm (**B**); 2 μm (**D**); 1 μm (**F, G, I**).

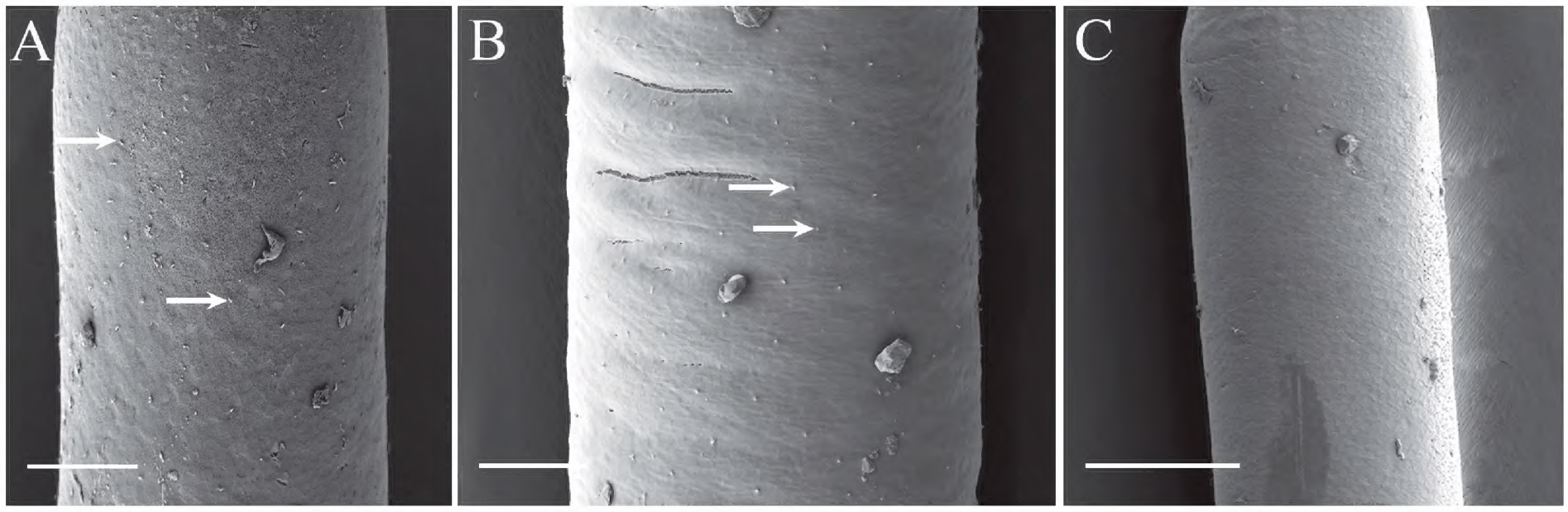

Figure 5. Mid-body of the *Gordius wulingensis* sp. nov. **A.** The mid-body near the anterior end, showing a higher density of bristles (arrows); **B.** The mid-body near the posterior end, also exhibiting a higher density of bristles (arrows); **C.** Absence of bristles in the mid-body region. Scale bars: 200 μm (**A, B**); 100 μm (**C**).

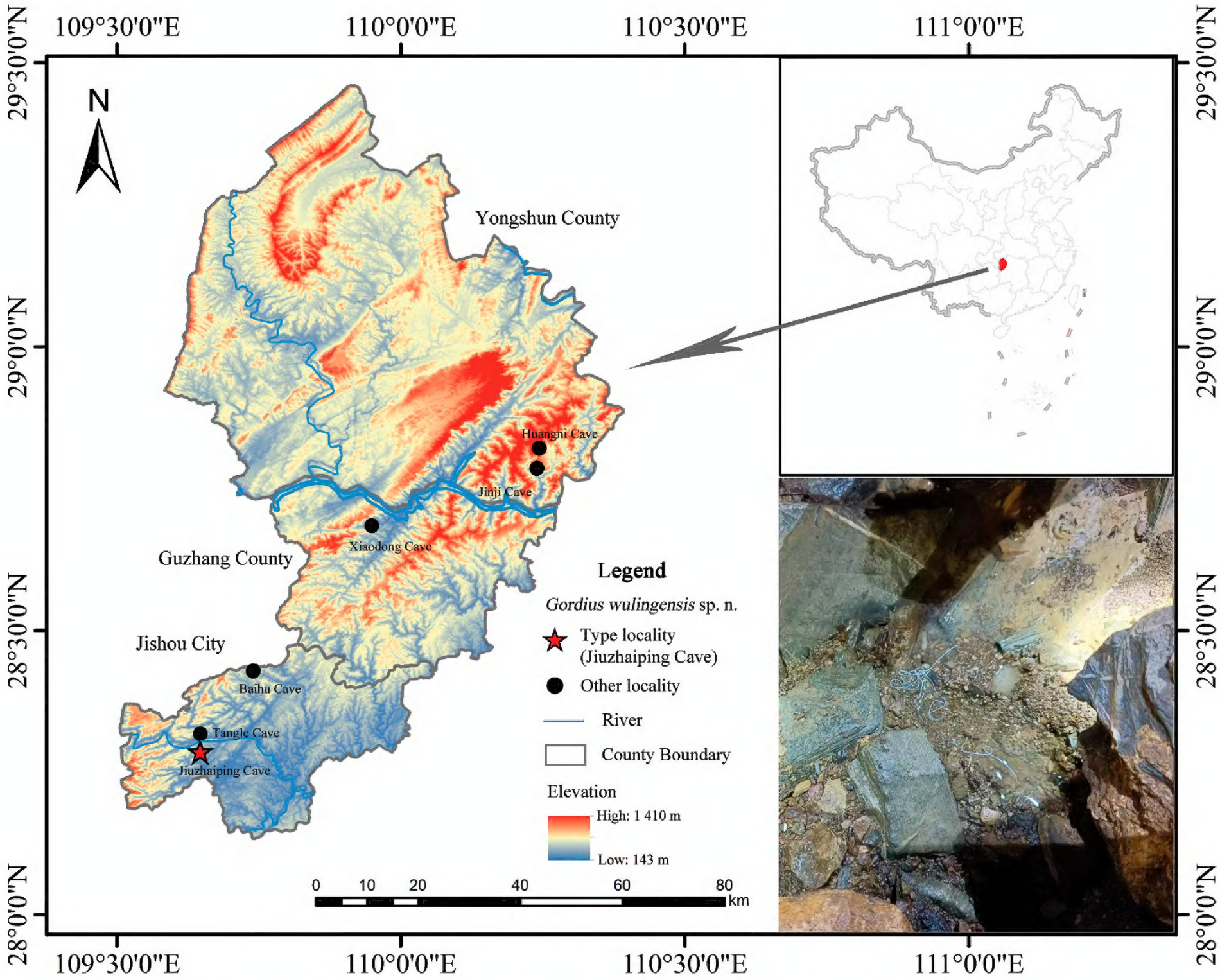

Figure 6. Collection sites of *Gordius wulingensis* sp. nov. along with photographic documentation of their habitats.

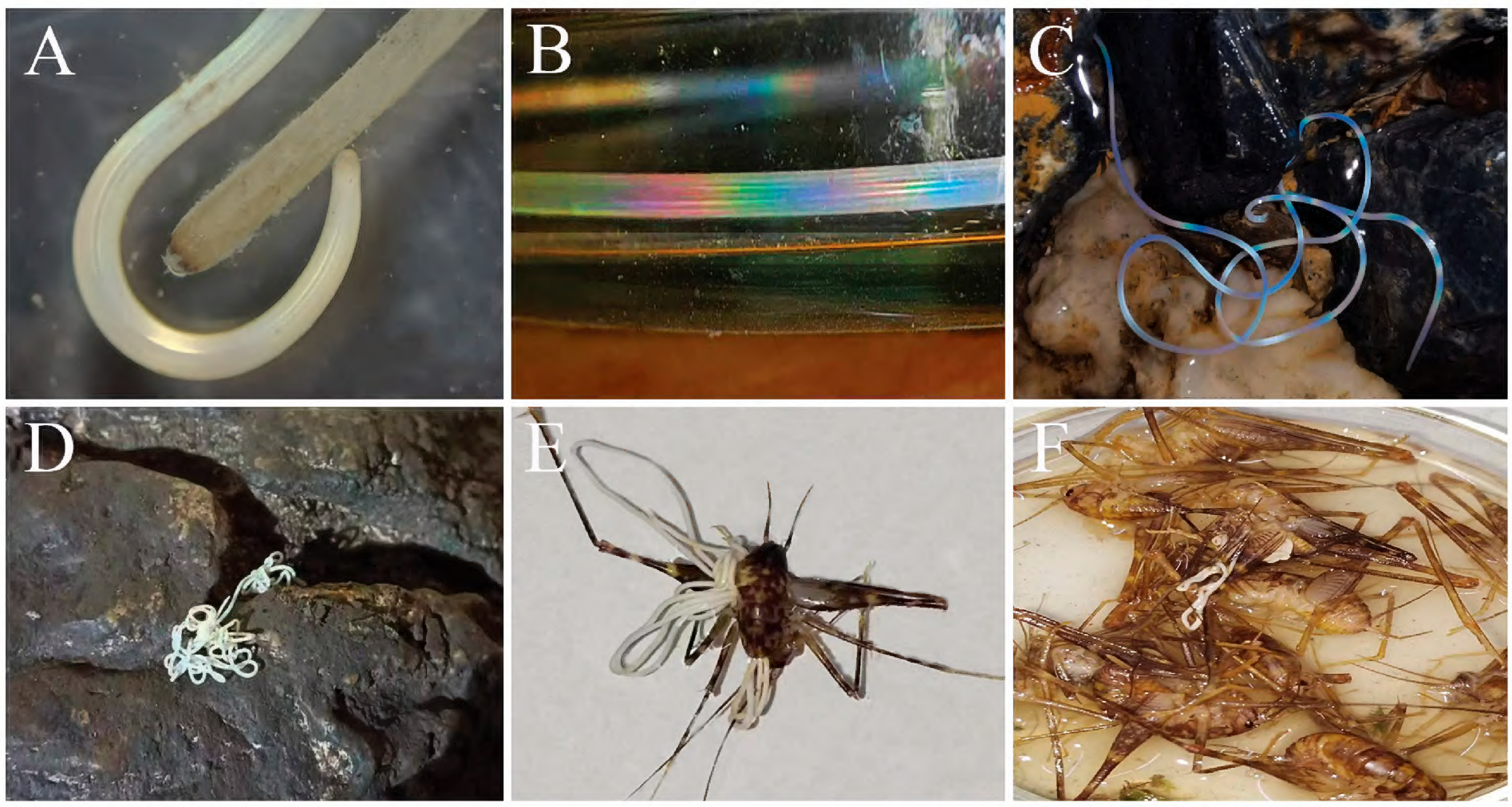

Figure 7. Observation of *Gordius wulingcavatuses* sp. nov. **A.** Comparison of the morphology of the anterior end and posterior end; **B.** A rainbow-like reflection on the body surface; **C.** Free-living adults observed in shallow water pools; **D.** Free-living adults found in moist soil environments; **E.** *Tachycines* sp. infested by an adult horsehair worm; **F.** The host being dissected.

Table 3. Uncorrected p-distance between Gordius/Acutogordius species based on COI gene.

No	Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*G. wulingensis*	**0.012**																						
2	*G.* cf. *robustus* (Clade 1)	0.225	**0.010**																					
3	*G.* cf. *robustus* (Clade 2)	0.219	0.143	**0.015**																				
4	*G.* cf. *robustus* (Clade 3)	0.264	0.182	0.171	**0.006**																			
5	*G.* cf. *robustus* (Clade 4)	0.235	0.146	0.155	0.115	**0.013**																		
6	*G.* cf. *robustus* (Clade 5)	0.229	0.103	0.138	0.151	0.143	**0.003**																	
7	*G.* cf. *robustus* (Clade 6)	0.227	0.170	0.151	0.161	0.149	0.163	**0.005**																
8	*G. terrestris*	0.221	0.144	0.167	0.170	0.155	0.147	0.151	**0.016**															
9	*G.* cf. *robustus* (Clade 8)	0.218	0.138	0.183	0.197	0.155	0.157	0.162	0.079	**0.021**														
10	*G. attoni*	0.214	0.145	0.184	0.204	0.171	0.153	0.168	0.156	0.153	**0.010**													
11	*G. balticus*	0.245	0.181	0.190	0.185	0.180	0.167	0.188	0.173	0.193	0.206	-												
12	*Acutogordius taiwanensis*	0.202	0.207	0.198	0.222	0.219	0.214	0.203	0.198	0.211	0.195	0.210	**0.002**											
13	*G.* sp. 1	0.243	0.214	0.216	0.236	0.223	0.222	0.188	0.213	0.209	0.215	0.240	0.243	-										
14	*G.* sp. 2	0.214	0.178	0.191	0.226	0.187	0.213	0.208	0.176	0.181	0.174	0.208	0.163	0.220	-									
15	*G.* sp. 3	0.221	0.183	0.175	0.225	0.202	0.208	0.206	0.191	0.194	0.186	0.188	0.130	0.248	0.160	-								
16	*G.* sp. 4	0.191	0.176	0.176	0.243	0.210	0.184	0.210	0.185	0.197	0.207	0.219	0.198	0.213	0.162	0.201	**0.011**							
17	*G.* sp. 5	0.236	0.242	0.253	0.286	0.249	0.254	0.263	0.224	0.242	0.216	0.258	0.263	0.255	0.218	0.238	0.204	-						
18	*G.* sp. 6	0.190	0.230	0.216	0.228	0.208	0.232	0.218	0.201	0.215	0.204	0.230	0.218	0.203	0.203	0.213	0.203	0.208	-					
19	*G.* sp. 7	0.162	0.208	0.206	0.255	0.216	0.214	0.207	0.217	0.215	0.208	0.230	0.250	0.230	0.208	0.220	0.186	0.225	0.188	-				
20	*G.* sp. 8	0.184	0.183	0.204	0.226	0.216	0.189	0.235	0.224	0.220	0.218	0.238	0.225	0.230	0.230	0.225	0.196	0.228	0.193	0.195	-			
21	*G. paranensis* (Clade1)	0.232	0.245	0.238	0.258	0.230	0.236	0.221	0.227	0.233	0.240	0.239	0.245	0.260	0.238	0.259	0.216	0.240	0.243	0.243	0.255	**0.038**		
22	*G. paranensis* (Clade2)	0.216	0.214	0.224	0.241	0.225	0.216	0.220	0.205	0.217	0.210	0.217	0.233	0.239	0.213	0.232	0.209	0.204	0.205	0.215	0.215	0.225	**0.007**	
23	*G. chiashanus*	0.237	0.183	0.191	0.178	0.190	0.168	0.186	0.159	0.167	0.179	0.199	0.215	0.213	0.215	0.204	0.204	0.244	0.214	0.233	0.230	0.216	0.212	**0.010**

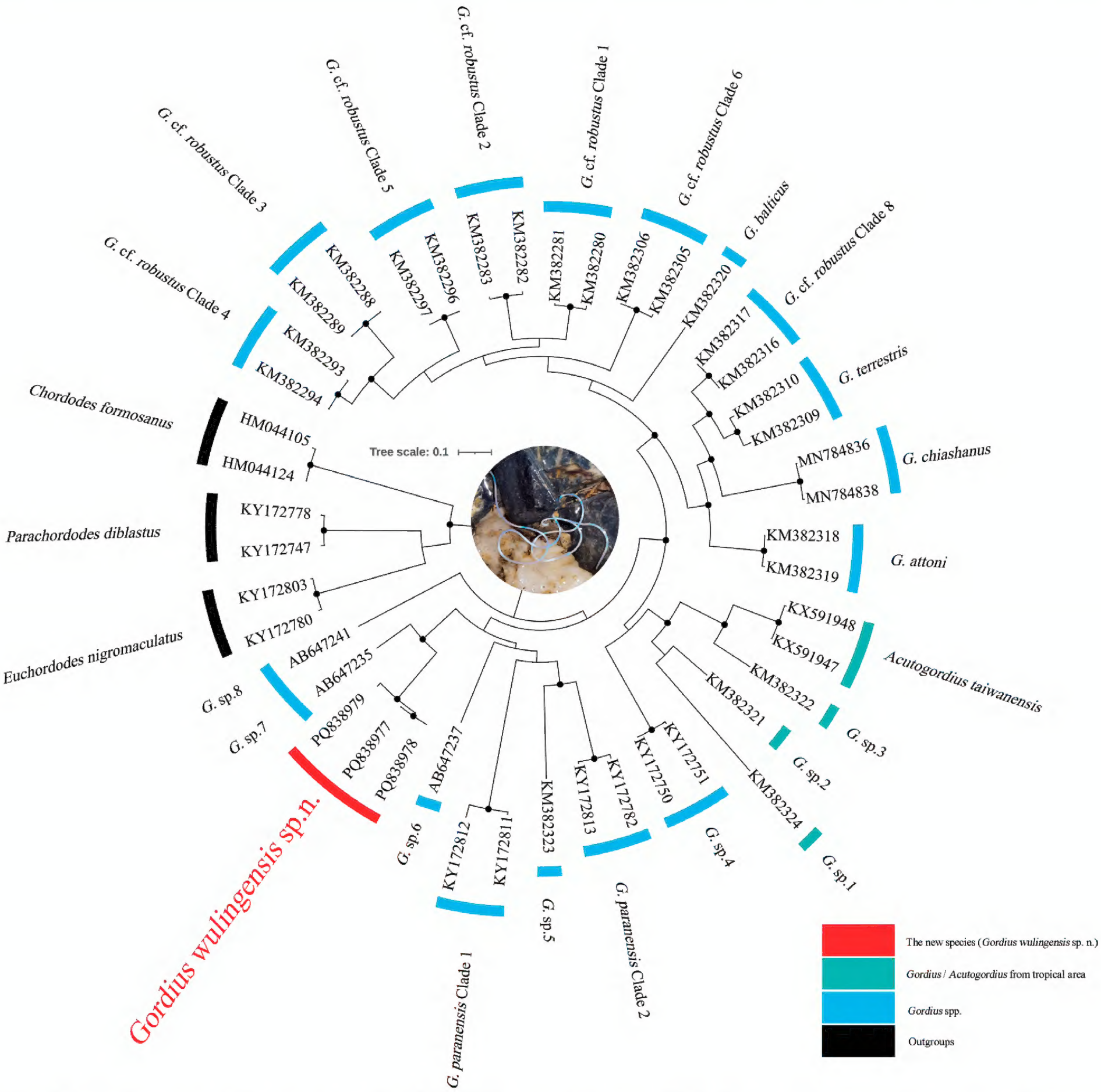

Figure 8. Phylogenetic relationship of *Gordius/Acutogordius* spp. restructured using COI partial sequences compared with *C. formosanus*, *E. nigromaculatus*, and *P. diblastus* as outgroups. Nodes marked with circles represent branches/nodes with maximum likelihood support values exceeding 90%.

Discussion

Identifying species within the genus *Gordius* is challenging due to their morphology, simple surface structures, and limited distinguishing features that can be utilized for taxonomic classification (Schmidt-Rhaesa 2010). Additionally, the cuticle is highly susceptible to environmental influences. Despite these challenges, only approximately 90 valid species of the genus *Gordius* have been described globally. However, research on the species diversity of horsehair worms in China, a country with vast territory, remains insufficient.

Morphological comparison

To date, no specimens of *Gordius wulingensis* have been found outside the cave in Wuling Mountains. The white color of the specimens suggests an adaptation to living in karstic caves, but as there are other white or lightly colored species living outside of caves (see below), we hesitate to call this species truly cavernicolous. We will continue to investigate the distribution of hairworm in this area in the future, inside and outside of the caves.

The body color of free-living horsehair worms can vary from nearly white to dark brown (Schmidt-Rhaesa

2010). The juvenile, parasitic stages of hair worms are characterized by a white appearance, which gradually turns fully brown after 30 days (in *Paragordius varius*, Schmidt-Rhaesa 2005). Specimens of *Gordius wulingensis* described in this study are entirely white. Some other species also exhibit a predominantly white coloration, including *Gordius difficilis* (white in females and yellowish-brown in males), *Gordius terrestris*, and *Gordius villoti*, which ranges from white to dark brown but are actually mainly brown (Valvassori et al. 1988; Bolek and Coggins 2002; Anaya et al. 2019). The cuticle around the cloacal opening and the postcloacal crescent in *Gordius wulingensis* are pigmented browner than the surrounding cuticle. *Gordius pararanensis* exhibits a brown body coloration with a darker crescent around the cloacal opening compared to the rest of its body (Schmidt-Rhaesa et al. 2000). *Gordius chiashanus* lacks such color differentiation (Chiu et al. 2020). This suggests that only certain species within the genus *Gordius* exhibit such color differences.

Although all species of the genus *Gordius* appear to possess "collar" and "cap" structures at the anterior end, the dark-brown collar may blend into the dark-brown body color, making the boundary between the "collar" and "cap" unclear (Schmidt-Rhaesa 2010).

In *Gordius wulingensis*, bristles are concentrated at the tips of the inner lobes. Some species in the genus *Gordius* have such concentrations of bristles in this position; for example, in *G. helveticus* from Switzerland, bristles are concentrated in a dense patch posterior to the tips of the postcloacal crescent (Schmidt-Rhaesa 2010), while *G. karwendeli* from Germany and *G. terrestris* from the United States both exhibit a long row of bristles concentrated medially (Schmidt-Rhaesa 2010; Anaya et al. 2019). *Gordius chiashanus* has densely distributed bristles on the top and inside of the tail leaf tip (Chiu et al. 2020).

Areoles on the inner wall of the male cloacal opening were first reported in an unidentified species of the genus *Gordius* (Schmidt-Rhaesa 2013). Subsequently, pebble-like areoles on the inner wall of the male cloacal opening were observed in *G. chiashanus* (Chiu et al. 2020). The cloacal areoles of *Gordius wulingensis* are honeycomb-like, differing from the areoles of the aforementioned species.

Gordius wulingensis is also distinguished by longitudinal ridges on both the ventral and dorsal surfaces, a morphological feature absent in other *Gordius* species.

Phylogenetic relationships of *Gordius wulingensis* sp. nov.

According to the available COI gene data for horsehair worms presented in the genetic distance table, the closest relatives identified were *Gordius* sp. 7 (KW-2011-A), *Gordius* sp. 6 (KW-2011-B), and *Gordius* sp. 8 (KW-2011-D) from Japan, with genetic distances from 0.162 to 0.190. *Gordius* sp. 5 (N297B) is distributed in New Mexico, USA, with a genetic distance ranging from 0.236 (Table 3). Due to the lack of specific taxonomic information for this unidentified species, morphological comparisons are challenging (Sato et al. 2012; Hanelt et al. 2015).

In the phylogenetic tree, *Gordius wulingensis* shares a sister-group relationship with *G. paranensis*, with a genetic distance ranging from 0.216 to 0.232. *Gordius paranensis* is distributed in New Zealand and South America, but the specimens included in this analysis (clade 1 and 2) are from New Zealand (Tobias et al. 2017). *Gordius paranensis* is a very characteristic species with a semicircular row of bristles anterior to the cloacal opening, a character not present in *G. wulingensis*. In summary, the specimens probably most closely related to *G. wulingensis* span a wide geographic area and do not shed much light on ancient distribution routes.

Life history

Gordius wulingensis can be found year-round within the caves investigated by us. Free-living adults are present in small numbers throughout the year but are more frequently encountered from October to December and July to August. The typical lifespan of free-living adult horsehair worms is 2 to 8 weeks (Bolek and Coggins 2002), but *Gordius wulingensis* has survived for over three months under laboratory conditions. These findings suggest that, in undisturbed natural karstic caves, adult horsehair worms may survive for more than three months.

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